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IN VIVO ANTIANXIETY ACTIVITY OF CALENDULA OFFICINALIS AERIAL PARTS IN MICE

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ABSTRACT

Traditionally, all parts of *Calendula officinalis* have been used for treating various diseases including anxiety disorders. Thus, we focused our study to investigate the antianxiety potential of *C. officinalis* aerial parts extracts and fractions using elevated plus maze (EPM) model. Extracts of *C. officinalis* aerial parts were prepared using solvents of increasing polarity (petroleum ether (60-80°C), chloroform, methanol and water) and were subjected to antianxiety activity on mice at the doses of 100, 200, 400 mg/kg, po. 200 mg/kg of *C. officinalis* methanol extract produced antianxiety effect comparable to reference compound, diazepam (2 mg/kg, po). Bioactive methanol extract of aerial parts *C. officinalis* was fractionated using ethyl acetate and found that it exhibits antianxiety activity and statistically equivalent to reference drug. Further, fraction was subjected to column chromatography in order to isolate sub-fractions responsible for antianxiety effect. Sub-fractions obtained were evaluated for the antanxiety activity in mice, among all only sub-fraction 4 (F_4) was found active, and compound isolated (CO_1) from the sub-fraction $F_{4.3}$ exhibited significant antianxiety activity at the dose of 6 mg/kg, po. Altogether, we can conclude that the results of *C. officinalis* aerial parts validate its traditional claim in the treatment of anxiety disorders.

KEYWORDS: *Calendula officinalis*, anxiety, methanol extract, chromatography, traditional, mice.

INTRODUCTION

Calendula (Family:Asteraceae) is a genus of perennial or annual herb worldwide including 25 species which are mainly distributed throughout southwestern Asia, western Europe, Macaronesia and the Mediterranean region. These are often known as marigolds.[1-3] Calendula was a natural drug therefore used traditionally in the treatment as an anti-spasmodic, anti-worm and diuretic.[4] Leaves and flowers of *C. officinalis* were considered as a natural herb and used as.

Diaphoretic, stimulant, emmenagogue, antispasmodic and also in the treatment of measles and smallpox and metrorrhagia.[5,6] whereas in India ointment of florets used in treating herpes, wounds, frostbite, ulcers, skin damage and scars.[7-9]

Traditional use of *C. officinalis* in treating inflammations, gastrointestinal ulcers and dysmenorrheal was revealed by a survey of ethnopharmacologic records.[10] Dried flower heads of *Calendula* have been used as antipyretic, antitumor, for cicatrizing wounds and also as a diuretic and diaphoretic.[11] *C. officinalis* also used topically used as a antifungal and antiseptic for treating wounds, sprain, conjunctivitis and marks.[12] Infusion of *Calendula* is used in the treatment of chronic infections and act as a cleansing and detoxifying herb.[13] In homoeopathic medicine, its mother tincture is used for the mental tension and insomnia treatment.[14]

In Ayurvedic and Unani System of Medicine, leaves and flowers of *C. officinalis* are considered for the treatment of antimicrobial, anti-inflammatory, antipyretic and antiepileptic.[15] In traditional and homoepathic medicine, whole plant used in the treatment of menstrual irregularities, duodenal ulcers, hemorrhoids and varicose veins.[16] In the middle ages, flowers of *C. officinalis* have been used for snake bites and liver obstruction. In 18th century; this plant was considered the best remedy for red eyes, jaundice and headache.

C. officinalis also included as part in the treatment of dry skin, bee stings and foot ulcers.[17] Essential oil extracted from the *C. officinalis* is used for soothing central nervous system and also act as a wound healer.[18]

After chemical research, various biologically active chemical constituents reported in *C. officinalis* include triterpenoids glycosides, flavonoids, sesquiterpenes glycoside, phenolic

acids, carotenoids, hydroxycoumarins, Volatile oils and fatty acids. The objective of the present study was to evaluate potential antianxiety activity of the *C. officinalis* aerial parts.

MATERIAL AND METHODS

Plant material and reagents

Aerial parts of *C. officinalis* were procured from the Himalaya Herbs Store, Saharanpur, UP, India. Identified by Head, Raw Materials, Herbarium & Museum at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi vide letter dated 19.10.2014 bearing number NISCAIR/RHMD/Consult/-2010-11/1431/29. Petroleum ether (60–80^{o}C), chloroform, methanol and ethyl acetate used for extraction of plant material. Anisaldehyde (0.5%) was used thin layer chromatography (TLC) for preparing visualizing reagent. Diazepam used as reference drug for the antianxiety activity.

Preparation of extracts

C. officinalis aerial parts (100 g) were dried, powdered and successively soxhlet extracted with petroleum ether, chloroform, methanol and water in the order of increasing polarity and the extracts were preserved in vacuum desiccators containing anhydrous silica gel blue.

Animals

The experimental animals (Swiss albino mice: males; n=6), weighing about 20-25g were bred at the Central Animal House, L R Institute of Pharmcy, Solan. All mice were maintained under controlled environmental conditions (25±2^{o}C) with 12 h light- 12 h dark cycle, given standard feed, water *ad libitum* and were fasted for 4 h before use. Animal Ethical Committee, L R Institute of Pharmcy, Solan has approved the use of animal for carrying out the biological studies. All the experiments were carried out as per CPCSEA guidelines.

Acute toxicity studies

These were conducted following OECD 423 guidelines (OECD, 2001). Single oral dose (500, 1000 or 2000 mg/kg of various prepared extracts of *C. officinalis* aerial parts were administered to mice in different groups. The mice were kept under observation immediately to see the sign of toxicity during the first 0.5, 1, 2, 4, 8 and 12 h, and at every 24 h for 14 days. Various behavioral parameters, lethargy, amount of water, feed taken, tremors and death were observed. Results reported that none of the extracts exhibited signs of toxicity upto the dose of 2000 mg/kg, po.

Preparation of doses

Test doses (100, 200 or 400 mg/kg, po) of various extracts of *C. officinalis* aerial parts were prepared using simple syrup (IP)+Tween 80 in the ratio 95:5 (vehicle) and diazepam (2 mg/kg, po) as a reference drug was administer to mice 45 min prior to EPM study using tuberculin syringe fitted with oral canula in the volume ranging between 0.20-0.30 ml.

Experimental model to test anxiolytic activity

Antianxiety study of plant extracts was evaluated by using well eslablish animal model i.e. EPM. It is 25 cm elevated from the floor, consisting of two open arms (16×5 cm) and two closed arms (16×5×12 cm) and radiant open roof which form plus sign to observed anxiolytic behavious of animal.[19] individual animal was placed at the center of model with its head towards one of the open arms. During the 5-min experiment, behaviour of the mouse was recorded as: (a) the number of entries the open arms and (b) average time spent by the mouse in the open arms (average time = total time spent in open arms/number of entries in open arms). Test dose was administered orally using tuberculin syringe fitted with an oral canula and such adjusted that every animal gets its turn after 45 min of the dose administration. And we have to ensure that no external stimuli other than the height of plus-maze could invoke anxiety in the animals.

TLC finger print profile of methanol extract

Dried powder of aerial parts of *C. officinalis* (2 g) was packed in filter paper sachet, placed inside 100 ml round bottom flask, macerated for 30 min with methanol (50 ml) followed by reflux (30 min) on a boiling water bath. After cooling, the extract was decanted off. Solvent was recovered under reduced pressure using rotary evaporator. The dried residue was reconstituted in 5 ml volumetric flask using methanol. For optimizing the mobile phase, aliquots 2 µl of extract solution was applied on TLC glass plate and developed in different solvent systems. Thin layer chromatograms were visualized initially under UV light at 366 nm followed by spraying with 0.5% anisaldehyde followed by heating at 110°C for 10 min.

TLC finger print profile of methanol extract was obtained on precoated aluminium-based TLC plates, developed in toluene:chloroform:methanol (7.2:1), using CAMAG auto sampler and scanner under controlled conditions.

Preparation and fractionation of methanol extract

Methanol extract of dried *C. officinalis* aerial parts (500 g) was prepared. Dried methanol extract (100 g), suspended in water, was placed in three-necked round bottom flask. It was, then, partitioned with ethyl acetate (100 ml) by heating (50°C) for 30 min using rotamantle. The procedure was repeated five times. All the ethyl acetate fractions were pooled. Solvent from the pooled fraction and the remaining aqueous fraction was recovered under reduced pressure using rotary vacuum evaporator to get ethyl acetate soluble fraction (EASF) and ethyl acetate insoluble fraction (EAIF), respectively.

Column chromatography of EASF and sub-fractions

EASF (20 g) was subjected to column chromatography using silica gel. Elution was done with petroleum ether, chloroform and methanol, in appropriate proportions. A total of 150 fractions, each of 500 ml were collected and pooled on the basis of TLC profiles to get four fractions – F_1-F_4.

The F_4 (13 g) was subjected to column chromatography. The column was packed with silica gel in petroleum ether. Elution was done with petroleum ether, chloroform and methanol, in appropriate proportions. A total of 162 fractions, each of 250 ml, were collected and pooled on the basis of TLC profiles, to get three sub fractions $F_{4.1}$- $F_{4.3}$. A crystalline compound – CO_1 (2.67 g), was separated from concentrated solutions of the sub-fraction $F_{4.3}$.

Statistics

The results have been expressed as Mean ± Standard Error Mean (SEM). The test doses were compared among themselves, and also with standard and control by one way analysis of variance (ANOVA) followed by Student Neumann Keuls test.[20] Control group was also compared with the standard group.

RESULTS

Yield of extracts

After successive soxhlet extraction of *C. officinalis* aerial parts using solvents in increasing polarity viz. petroleum ether, chloroform, methanol and water extracts and yield was found to be 07.26%, 05.42%, 20.35% and 07.91% (w/w), respectively.

Acute toxicity studies

Among all the extracts of *C. officinalis* aerial parts, none of them was observed toxic up to a dose of 2 000 mg/kg.

Antianxiety activity of extracts

After oral administration of the extracts of *C. officinalis* aerial parts at the oral doses of 100, 200 or 400 mg/kg, diazepam (2 mg/kg) and the control (vehicle), the mean time spent by the mice in open arms shown in **Table 1**. Among the tested extracts, dose of 100 mg/kg, p.o. of methanol extract was observed to have maximum anxiolytic activity.

TLC finger print profile of methanol extract was obtained on precoated aluminium-based TLC plates, developed in toluene: chloroform: methanol (7.2:1), using CAMAG auto sampler and scanner under controlled conditions (**Table 2).**

Ethyl acetate fraction was obtained by further fractionation of bioactive methanol extract. Yields of EASF and ESIF were found to be 21.6 and 76.2 % (w/w), respectively and evaluated for antianxiety activity using EPM in mice. The mean number of entries and time spent in open arms of EPM were shown in **Table 3** at the doses 20 and 75 mg/kg, po, of *C. officinalis* aerial parts, diazepam (2 mg/kg, po) and the control (vehicle) and found that only EASF at the dose of 20 mg/kg significantly exhibited antianxiety with respect to control and the activity was equivalent to the reference drug.

EASF fraction was again fractionated by column chromatography; fraction yielded four sub-fractions (F_1–F_4), out of which only F_4 at a dose of 15 mg/kg exhibited significant antianxiety activity (Table 4). Four subfractions ($F_{4.1}$–$F_{4.3}$ and CO_1) were obtained from column chromatography of F_4 fraction and screened for antianxiety activity. Among them, isolated compound (CO_1) at a dose of 6 mg/kg, po, show significant increase in the number of entries (7.5 ± 0.10) and the time spent (12.5 ± 0.24 s) in the open arms (Table 5).

Table 1: Antianxiety activity of different extracts of *C. officinalis* aerial plants using EPM.

Treatment	Dose (mg/kg, po)	Number of entries in open arms (Mean^n±SEM)	Average time spent in open arms (sec) (Mean^n±SEM)
Vehicle	0.25 ml	3.2 ± 0.13^b	3.7 ± 0.23^b
Diazepam	2	7.5 ± 0.12^a	15.2 ± 0.32^a
Petroleum ether extract	100	2.7 ± 0.45^b	6.4 ± 0.34^b
	200	3.1 ± 0.77^b	2 ± 0.43^b
	400	3.5 ± 0.55^b	2.2 ± 0.24^b
Chloroform extract	100	2.5 ± 0.55^b	3.4 ± 0.81^b
	200	2.4 ± 0.55^b	2.1 ± 0.36^b

	400	2.9 ± 0.85 [b]	2.6 ± 0.45 [b]
Methanol extract	**100**	**5.9 ± 0.89 [b]**	**11.9 ± 0.31** [b]
	200	4.7 ± 0.85 [b]	10.1 ± 1.25 [b]
	400	3.8 ± 0.84 [b]	8.7 ± 0.73 [b]
Water extract	100	2.3 ± 0.45 [b]	3.3 ± 0.45 [b]
	200	2.8 ± 0.45 [b]	2.5 ± 0.51 [b]
	400	3.2 ± 0.77 [b]	3.3 ± 0.53 [b]

n=6; The data is expressed as mean±SEM; p<0.001, [a]: vs. control, [b]: vs. diazepam; one way ANOVA followed by Student Newmann Keul's test.

Table 2: Mobile phases employed for TLC of methanol extract of *C. officinalis* aerial plants.

Sr. no.	Mobile phase	Proportions
1	Chloroform:Methanol	8:2
2	Chloroform:Methanol	9:1
3	Toluene:Ethyl acetate	9:1
4	Toluene:Ethyl acetate:Glacial acetic acid	7:2:1
5	Toluene: Glacial acetic acid	9:1
6	Propanol:Water	9:1
7	Propanol:Water	8:2
8	Toluene:Chloroform:Methanol	6:2:2
9	Toluene:Chloroform:Methanol	6:3:1
10*	Toluene:Chloroform:Methanol	7:2:1

*Optimum resolution

Table 3: Antianxiety activity of EASF and EAIF of methanol extract using EPM.

Treatment	Dose (mg/kg, po)	Number of entries in open arms (Mean[n]±SEM)	Average time spent in open arms (sec) (Mean[n]±SEM)
Vehicle	0.25 ml	3.2 ± 0.13[b]	3.7 ± 0.23[b]
Diazepam	2	7.5 ± 0.12[a]	15.2 ± 0.32[a]
EASF	**20**	**5.4 ± 0.15**[b]	**9.6 ± 0.32**[a,b]
EAIF	75	2.4 ± 0.12[b]	4.8 ± 0.21[b]

n=6; The data is expressed as mean±SEM; p<0.001, [a]: vs. control, [b]: vs. diazepam, One way ANOVA followed by Student Newmann Keul's test. EASF: ethyl acetate soluble fraction, EAIF: ethyl acetate insoluble fraction.

Table 4: Antianxiety activity of F_1-F_4 fractions of EASF.

Treatment	Dose (mg/kg, po)	Number of entries in open arms (Mean[n]±SEM)	Average time spent in open arms (sec) (Mean[n]±SEM)
Vehicle	0.25 ml	3.2 ± 0.13[b]	3.7 ± 0.23[b]
Diazepam	2	7.5 ± 0.12[a]	15.2 ± 0.32[a]
F_1	0.5	2.2 ± 0.14[b]	3.2 ± 0.31[b]

F_2	1.5	2.0 ± 0.16^{b}	2.8 ± 0.25^{b}
F_3	3	2.7 ± 0.19^{b}	3.1 ± 0.33^{b}
F_4	15	$5.5 \pm 0.13^{a,b}$	$10.2 \pm 0.21^{a,b}$

n=6; The data is expressed as mean±SEM, p<0.001, [a]: vs. control, [b]: vs. diazepam; One way ANOVA followed by Student Newmann Keul's test. F_1-F_4: fractions of EASF.

Table 5: Antianxiety activity of various sub-fractions using EPM.

Treatment	Dose (mg/kg, po)	Number of entries in open arms (Mean[n]±SEM)	Average time spent in open arms (sec) (Mean[n]±SEM)
Vehicle	0.25 ml	3.2 ± 0.13^{b}	3.7 ± 0.23^{b}
Diazepam	2	7.5 ± 0.12^{a}	15.2 ± 0.32^{a}
$F_{4.1}$	2	2.6 ± 0.20^{b}	2.3 ± 0.33^{b}
	4	2.5 ± 0.18^{b}	2.0 ± 0.36^{b}
$F_{4.2}$	5	2.4 ± 0.14^{b}	1.8 ± 0.30^{b}
	10	2.6 ± 0.19^{b}	2.6 ± 0.27^{b}
$F_{4.3.1}$	4	3.0 ± 0.12^{b}	2.3 ± 0.31^{b}
	8	2.6 ± 0.19^{b}	2.6 ± 0.27^{b}
CO_1	3	4.0 ± 0.12^{b}	7.8 ± 0.31^{b}
	6	7.5 ± 0.10^{b}	12.5 ± 0.24^{b}

n=6; The data is expressed as mean±SEM, p<0.001, [a]: vs. control, [b]: vs. diazepam; One way ANOVA followed by Student Newmann Keul's test. $F_{4.1}$, $F_{4.2}$, $F_{4.3}$, $F_{4.3.1}$ and CO_1 sub-fractions obtained after column chromatography of F_4.

DISCUSSION

Affective disorders, especially anxiety and depression, are among the most common disorders.[21] Both are associated with diminished health status and substantially lower health-related quality of life that persists over time.[22-23] To date, benzodiazepines (BZDs) are the most widely for psychiatric disorders.[24-25] but the adverse effects caused by BZDs are a matter of concern. Thus, drugs obtained from natural sources are emerging as adjuvant therapies in the treating the various diseases[26] and psychiatric disorders,[27] as these are relatively free from adverse effects.

Despite the widespread use of *C. officinalis* aerial parts for treating mental disorders, no systematic studies have been carried out for evaluating its antianxiety activity. The present investigation demonstrates that among the various extracts of *C. officinalis* aerial parts, methanol extract at the dose of 100 mg/kg, po exhibited significant anxiolytic effect with respect to vehicle and equivalent to the reference drug. Bioactivity-guided isolation of the methanol extract yielded anxiolytic fraction F_4 at the dose of 15 mg/kg, po which was at par

with that of diazepam. The column chromatography of bioactive fraction of F_4 yields three sub-fractions $F_{4.1}$-$F_{4.3}$ and from $F_{4.3}$ sub-fraction yields CO_1 as a crystalline compound. This crystalline compound was separately suspended in a suitable vehicle, and evaluated for the antianxiety activity compared with that observed in the control group as well as with the group treated with the reference anxiolytic drug diazepam. Maximum anxiolytic activity was found to be in CO_1 at the dose of 6 mg/kg, po which was at par with that of diazepam as is evident from statistical equivalence between the results of this dose and that manifest by diazepam.

CONFLICTS OF INTEREST

All authors have none to declare

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